

Surface Coatings Determine Cytotoxicity and Irritation Potential of Quantum Dot Nanoparticles in Epidermal Keratinocytes

Jessica P. Ryman-Rasmussen¹, Jim E. Riviere¹ and Nancy A. Monteiro-Riviere¹

Quantum dot (QD) nanoparticles have potential applications in nanomedicine as drug delivery vectors and diagnostic agents, but the skin toxicity and irritation potential of QDs are unknown. Human epidermal keratinocytes (HEKs) were used to assess if QDs with different surface coatings would cause differential effects on HEK cytotoxicity, proinflammatory cytokine release, and cellular uptake. Commercially available QDs of two different sizes, QD 565 and QD 655, with neutral (polyethylene glycol (PEG)), cationic (PEG-amine), or anionic (carboxylic acid) coatings were utilized. Live cell imaging and transmission electron microscopy were used to determine that all QDs localized intracellularly by 24 hours, with evidence of QD localization in the nucleus. Cytotoxicity and release of the proinflammatory cytokines IL-1 β , IL-6, IL-8, IL-10, and tumor necrosis factor- α were assessed at 24 and 48 hours. Cytotoxicity was observed for QD 565 and QD 655 coated with carboxylic acids or PEG-amine by 48 hours, with little cytotoxicity observed for PEG-coated QDs. Only carboxylic acid-coated QDs significantly increased release of IL-1 β , IL-6, and IL-8. These data indicate that QD surface coating is a primary determinant of cytotoxicity and immunotoxicity in HEKs, which is consistent across size. However, uptake of QDs by HEKs is independent of surface coating.

Journal of Investigative Dermatology (2007) **127**, 143–153. doi:10.1038/sj.jid.5700508; published online 10 August 2006

INTRODUCTION

Semiconductor nanocrystals, or quantum dots (QDs) have great potential for use as drug delivery, diagnostic, and imaging agents in biomedicine and as semiconductors in the electronics industry. QDs are a diverse class of engineered nanostructures that are highly variable in chemical composition, size, and shape and are frequently multi-layered. The center is comprised of a heterogeneous, colloidal core of inorganic atoms (e.g., CdSe, CdTe, InAs, GaN) that is frequently surrounded by a shell or “cap”, such as ZnS, that can reduce leaching of core metals (Derfus *et al.*, 2004) and attenuate fluorescence intermittency, or “blinking” (Nirmal *et al.*, 1996). Surface coatings in one or more layers are often applied to the core/shell complex to customize QDs for different applications, such as increased solubility in biological media or bioconjugation to antibodies or receptor ligands for “targeted” drug delivery and diagnostics

(Michalet *et al.*, 2005). Nanoscale materials possess novel properties as a direct consequence of size. This may have unique consequences for toxicology (Monteiro-Riviere and Ryman-Rasmussen, 2006). The hallmark nanoscale property of QDs is quantum confinement, in which the distance between an excited electron in the conduction band and the corresponding vacant hole in the valence band (the “Bohr radius”) is greater than the size of the nanocrystal core, which results in fluorescence at size-dependent wavelengths. The excitation range of QDs is broad but the fluorescence is discreet and resistant to photobleaching, making QDs easily detectable and amenable to multicolor imaging studies and tracking in live cells.

A seminal study demonstrated that targeted CdSe core/ZnS shell QDs could be internalized by HeLa cells and tracked in live cells for more than 10 days with no morphological signs of toxicity (Jaiswal *et al.*, 2003). This study fueled a great deal of optimism that QDs were non-toxic at doses suitable for long-term imaging studies and drug delivery. Therefore, many synthetic efforts were focused in these directions. There are increasing reports of QD cytotoxicity in the literature (Hardman, 2006), none of which address skin. Recently, we reported that exposure of skin to commercially available QDs differing in core/shell shape, hydrodynamic size, and surface coatings resulted in the penetration of the intact stratum corneum barrier with localization of QDs in the underlying epidermal and dermal layers as early as 8 hours after topical application (Ryman-Rasmussen *et al.*, 2006). This indicated that skin is a potential route of exposure to QDs.

¹Center for Chemical Toxicology Research and Pharmacokinetics, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA

Correspondence: Dr Nancy A. Monteiro-Riviere, Center for Chemical Toxicology Research and Pharmacokinetics, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, North Carolina 27606, USA. E-mail: nancy_monteiro@ncsu.edu

Abbreviations: HBSS, Hank's balanced salt solution; HEK, human epidermal keratinocyte; KGM-2, keratinocyte growth medium-2; PEG, polyethylene glycol; QD, quantum dot; TEM, transmission electron microscopy; MTT, 3-[4,5]dimethylthiazol-2,5 diphenyltetrazolium bromide

Received 14 April 2006; revised 2 June 2006; accepted 20 June 2006; published online 10 August 2006

The present study utilized primary human neonatal epidermal keratinocytes (HEKs), an established *in vitro* model for epidermal toxicity, to determine the cytotoxic and inflammatory potential of QDs in skin. This study was designed to test the hypothesis that QDs would be differentially taken up by HEKs and cause cytotoxicity and inflammatory cytokine release in a manner dependent on surface coating. Soluble QDs of two sizes, QD 565 and QD 655, were obtained from a single, commercial source. Both QD 565 and QD 655 were obtained with three different surface coatings: polyethylene glycol (PEG), PEG-amines, or carboxylic acids. We used laser scanning confocal microscopy of live keratinocytes and transmission electron microscopy (TEM) to verify QD uptake 24 hours after administration. Cytotoxicity was assessed by cell viability assays with thiazolyl blue tetrazolium bromide (3-[4,5]dimethylthiazol-2,5 dephenyltetrazolium bromide (MTT)) at 24 and 48 hours. Inflammatory potential was assessed at 24 and 48 hours by measuring the release of the proinflammatory cytokines IL-1 β , IL-6, IL-8, IL-10, and tumor necrosis factor- α that are often used as markers for cutaneous irritation.

RESULTS

Laser scanning confocal microscopy of QDs in live HEKs at 24 hours

We observed no overlap in the emission channels for QD 565 (green) and QD 655 (red) in trials with fixed keratinocytes

treated with 2 nM QD 565 or QD 655, which indicated that dual-label imaging experiments were valid at this concentration, and autofluorescence in the absence of QDs was negligible (data not shown). QDs of both sizes and all coatings were localized intracellularly by 24 hours (Figure 1). Punctate staining of PEG-coated QD 565 and QD 655 (Figure 1, top row) was observed in the cytoplasm, at the periphery of the nucleus, and in the nucleus (QD 565). There was no evidence of PEG-coated QD 565 and QD 655 colocalization (orange), although there were instances of individual cells that contained both green and red punctae. Approximately 50% of HEK cells in any visual field contained PEG-coated QDs. PEG-amine-coated QD 565 and QD 655 (Figure 1, middle row) were observed as very large green (QD 565) or red (QD 655) punctae in the cytoplasm. PEG-amine-QD-treated cells contained QD 565 or QD 655, QD 565 and QD 655 in separate regions of the same cell, or colocalized QD 565 and QD 655 (orange). Staining for PEG-amine-coated QD 565 and QD 655 was sporadic, as the majority of HEK cells in the culture dishes did not stain. Carboxylic acid-coated QD 565 and QD 655 demonstrated very different patterns of cytoplasmic localization than QDs coated with PEG or PEG-amines (Figure 1, bottom row). A majority of cells throughout the culture dish demonstrated diffuse, punctate, staining for both QD 565 and QD 655, with large and discrete punctae containing colocalized QD 565 and QD 655 (orange). Intracellular staining of carboxylic acid-coated QD 565 and QD 655 was also observed.

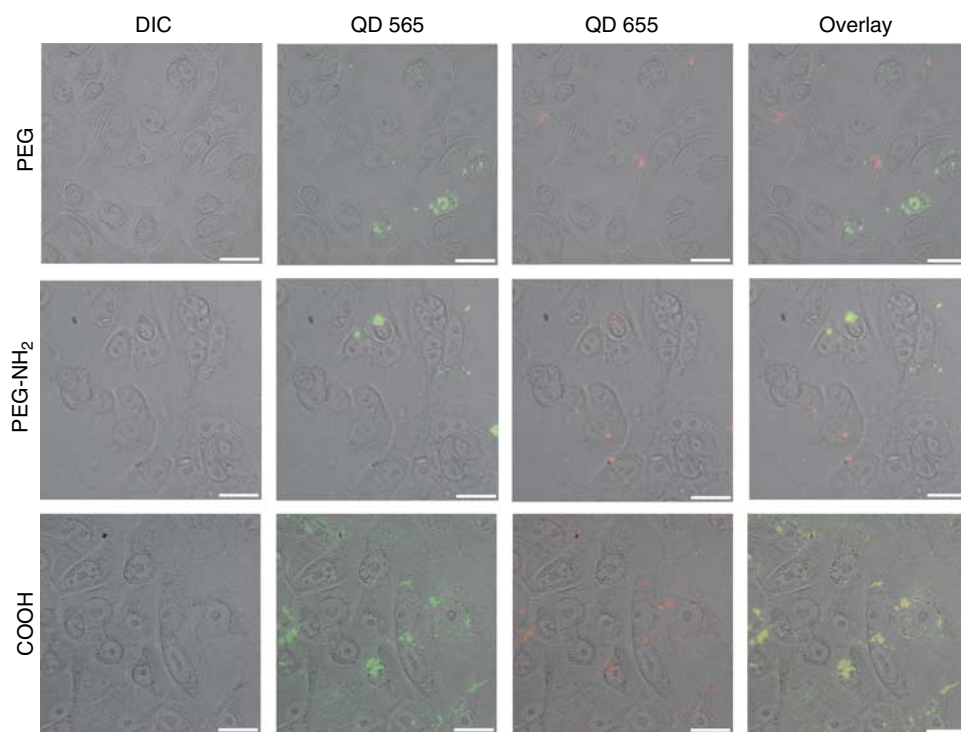


Figure 1. Uptake of QDs by live keratinocytes at 24 hours. HEKs were treated for 24 hours with QD 565 and QD 655 of the same coating (PEG, PEG-NH₂, or COOH) at a final concentration of 2 nM each and were imaged by live cell laser scanning confocal microscopy. Columns (left to right) show the DIC channel, QD 565 emission channel, QD 655 emission channel, and an overlay of all three channels. Rows indicate QD coating. QD 565 are pseudo-colored green, QD 655 are pseudo-colored red, and colocalized QD 565 and QD 655 are orange. Bar = 25 μ m. Data are representative of three similar, independent experiments.

TEM of QDs in HEKs at 24 hours

QDs of both sizes and all coatings were intracellularly localized by 24 hours. In all cases, QDs were usually found agglomerated, both in membrane-bound, vacuolar compartments (Figure 2a) or free in the cytoplasm (Figure 2b). Occasionally with the PEG and PEG-amine-coated QD 565, we observed lipid droplets that were peppered with QDs in the interior and more dense along the periphery (Figure 2c). We also observed several instances for PEG-coated QD 565 and QD 655 and carboxylic acid-coated QD 565 in which QDs were agglomerated within the intranuclear region of the cells. (Figure 2d).

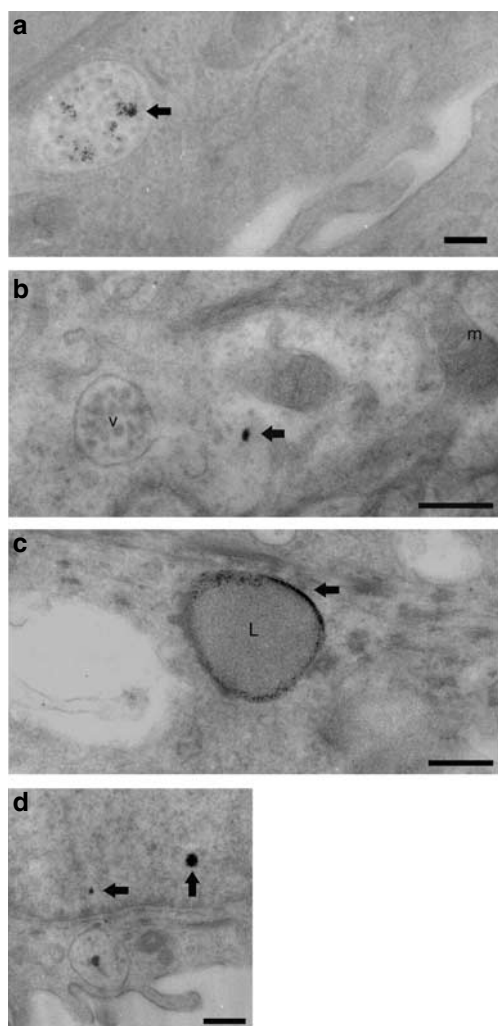


Figure 2. TEM of QD in keratinocytes at 24 hours. HEKs were treated for 24 hours with 2 nM QD 565 or QD 655 coated with PEG, PEG-amines, or carboxylic acids. Arrows indicate QDs. (a) Example of QDs in a cytoplasmic vacuole. Carboxylic acid-coated QD 655 is shown. (b) Example of unsequestered QDs free in the cytoplasm. Note the vacuole (v) and mitochondria (m). PEG-amine-coated QD 655 is shown. (c) Example of lipid droplet (L) with QD staining along the periphery of the membrane. PEG-coated QD 655 is shown. (d) Example of QD aggregates within the nucleus. PEG-coated QD 655 is shown.

MTT cell viability assays at 24 and 48 hours

No effects of 5% (v/v) borate vehicle of pH 8.3 or 9.0 were seen on HEK viability at 24 or 48 hours (Figure 3a).

No differences in HEK viability were observed for three concentrations of PEG-coated QD 565 at 24 or 48 hours (Figure 3b). No differences were observed for PEG-coated QD 655 at 24 hours. At 48 hours, however, there was a moderate (20%) but significant decrease in MTT viability at 20 nM compared to vehicle-treated controls and lower concentrations.

No differences in viability at any of the three concentrations were observed for PEG-amine-coated QD 565 at 24 hours (Figure 3c). At 48 hours, however, viability decreased by 40% at the highest concentration compared to vehicle-treated controls and lower concentrations. As cytotoxicity was present at 48 hours but not 24 hours, we statistically compared viability at 24 and 48 hours at 20 nM and found a significant effect of time on viability. Similarly, for QD 655 coated with PEG-amine, there were no dose-dependent differences in viability at 24 hours. However, at 48 hours, viability at 20 nM was decreased to 40% compared to vehicle-treated controls and lower concentrations. There was also a significant effect of time on the cytotoxicity of PEG-amine-coated QD 655 at 20 nM between 24 and 48 hours.

Unlike QD 565 coated with PEG or PEG-amines, there were significant differences in HEK viability at 24 hours for carboxylic acid-coated QD 565 (Figure 3d). HEKs treated at the highest of the three concentrations (20 nM) showed a 40% decrease in viability compared to vehicle-treated controls and lower concentrations. These differences remained at 48 hours. Similarly, QD 655 coated with carboxylic acids decreased HEK viability by 40% at 24 hours compared to vehicle-treated controls and lower concentrations. These differences remained at 48 hours, where viability was decreased by another 30%. This decrease in viability between 24 and 48 hours was significant and indicated an effect of time on cytotoxicity. As the decrease in viability between 24 and 48 hours for carboxylic acid-coated QD 565 was smaller than QD 655, we conducted statistical comparisons to see if there was an effect of size on the cytotoxicity. Comparison at 24 hours revealed no size differences. However, viability for QD 655 was significantly decreased relative to QD 565 at 48 hours, indicating a modulatory role for size in cytotoxicity.

Cytokines

For all detectable cytokines, there were no effects of 5% (v/v) borate vehicle pH 8.3 or 9.0 at 24 or 48 hours.

IL-10 and tumor necrosis factor- α

IL-10 and tumor necrosis factor- α release were not detectable for QD 565 and QD 655 of any coating at 24 or 48 hours within the low range of the multiplex assay, which had a threshold of 0.78 pg/ml.

IL-1 β

The basal, control levels of IL-1 β in the culture medium at 24 hours averaged 1.1 ± 0.2 and 2.6 ± 0.7 pg/ml at maximal

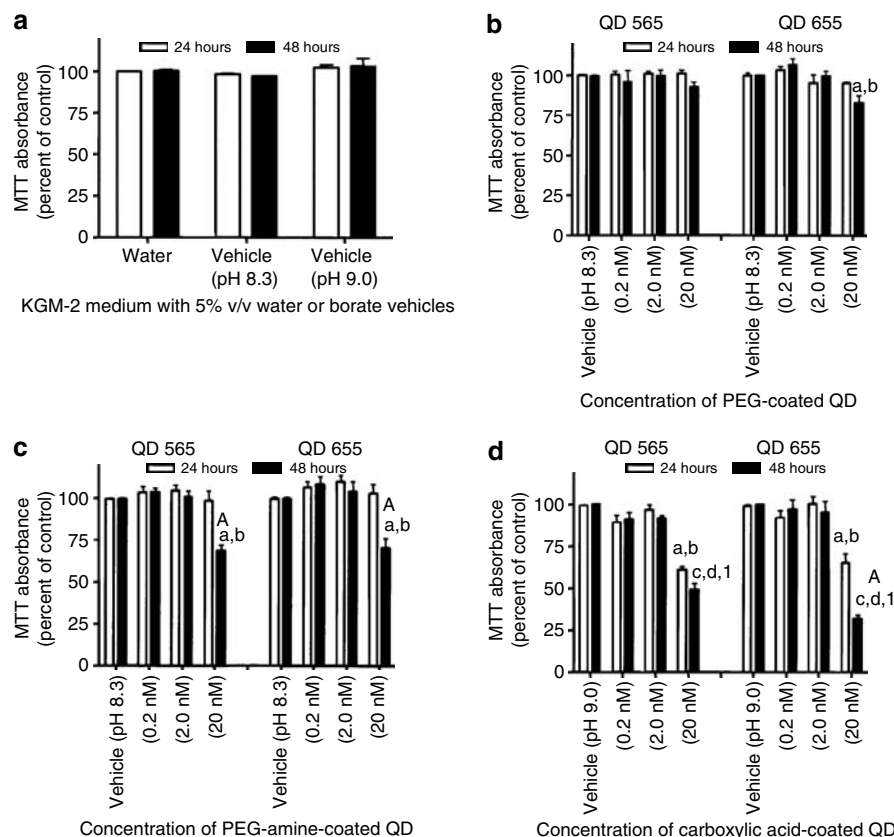


Figure 3. MTT cell viability assays at 24 and 48 hours. (a) Effect of 5% (v/v) water or borate vehicle on MTT viability. (b–d) HEKs were treated with 5% (v/v) vehicle, QD 565, or QD 655 at 0.2, 2, or 20 nM. (b) PEG-coated QDs; (c) PEG-amine-coated QDs; and (d) Carboxylic acid-coated QDs. Lower case a and b: $P < 0.05$ compared to (a) vehicle-treated controls or (b) lower doses within each QD size. Lower case c and d: same, but at 48 hours. Upper case A: an effect of time on viability between 24 and 48 hours when concentration and coating are held constant ($P < 0.05$). Number 1: a significant effect of size on viability when concentration, time, and coating are held constant ($P < 0.05$).

response to QDs. Basal levels at 48 hours averaged 2.6 ± 0.6 and 12.2 ± 2.6 pg/ml at maximal response to QDs (data not shown).

Effects of dose and coating on IL-1 β release were observed for QD 565. At 24 hours, there was over a 2-fold, dose-dependent increase in IL-1 β release for carboxylic acid-coated QD 565 (Figure 4b). IL-1 β release for this coating was also significantly elevated compared to PEG and PEG-amine coatings at either dose. These differences remained at 48 hours (Figure 4c). The magnitude of IL-1 β release for carboxylic acid-coated QD 565 did not change between 24 and 48 hours (Figure 4d).

Effects of coating and time on IL-1 β release were observed for QD 655. At 24 hours, there was over a 3-fold, dose-dependent increase in IL-1 β release for carboxylic acid-coated QD 655 (Figure 4b). This was significantly elevated compared to QD 655 coated with PEG or PEG-amines. At 48 hours, the magnitude of IL-1 β release carboxylic acid-coated QD 655 at the highest dose exceeded controls, the lower dose, and other coatings by over 5-fold (Figure 4c). Comparison of IL-1 β levels for 20 nM carboxylic acid-coated QD 655 at 24 and 48 hours revealed a significant effect of time on the magnitude of IL-1 β release for this coating and dose (Figure 4d).

At 48 hours, the difference in IL-1 β release for 20 nM carboxylic acid-coated QD 655 and QD 565 was greater than 2-fold (Figure 4c). Statistical analysis confirmed that this difference was significant, revealing an effect of size on IL-1 β release.

IL-6

The basal, control levels of IL-6 in the culture medium at 24 hours averaged 110 ± 30 and 220 ± 80 pg/ml at maximal response to QDs. Basal levels at 48 hours averaged 130 ± 50 and 260 ± 70 at maximal response to QDs (data not shown).

Effects of dose and coating were observed for QD 565. At 24 hours, IL-6 release for carboxylic acid-coated QD 565 at 20 nM was over 50% greater than control and the lower dose (Figure 5b). At 48 hours, 20 nM carboxylic acid-coated QD 565 was elevated 2-fold compared to controls, the lower dose, and PEG coating at either dose (Figure 5c). There was also a slight but significant dose-dependent elevation in IL-6 release for 20 nM PEG-amine-coated QD 565 compared to controls and the lower dose. IL-6 release for carboxylic acid-coated QD 565 did not significantly differ between 24 and 48 hours (Figure 5d).

IL-6 release for QD 655 was dependent on dose, coating, and time. Unlike QD 565, there were no effects of coating or

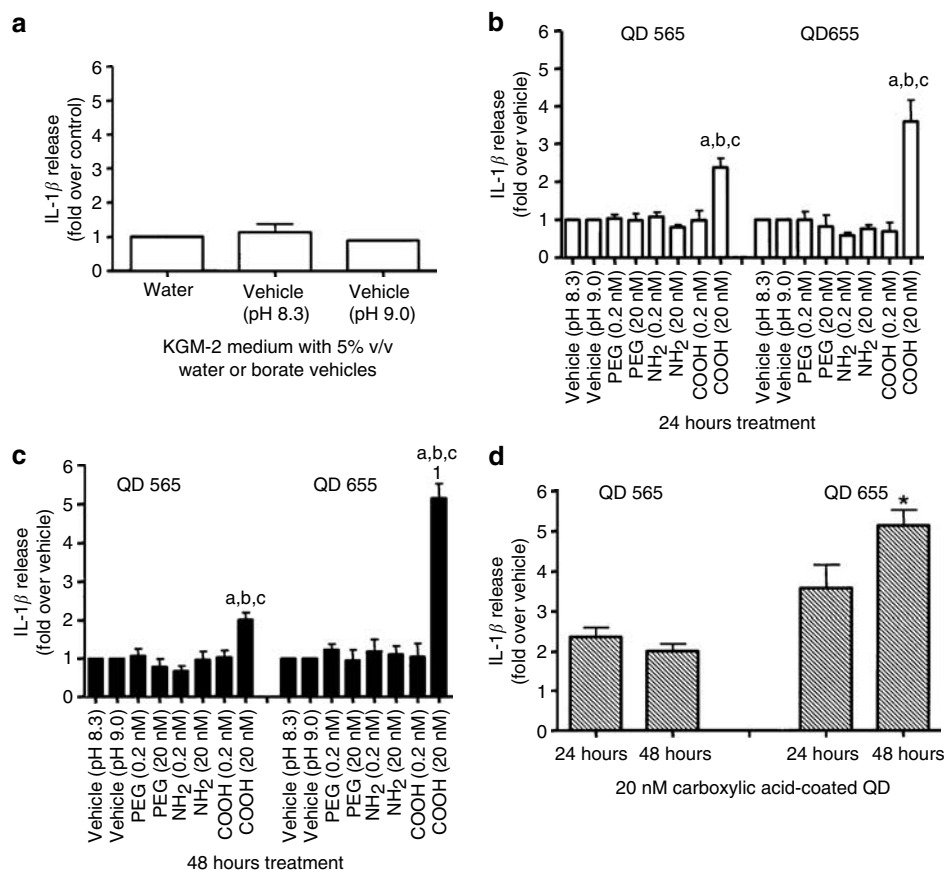


Figure 4. IL-1 β release at 24 and 48 hours. (a) Effect of 5% (v/v) water or borate vehicle on IL-1 β release at 24 hours. (b-d) HEKs were treated with 5% (v/v) of vehicle, QD 565, or QD 655 coated with PEG, PEG-amines, or carboxylic acids at 0.2 or 20 nM. (b) Twenty four hours after QD treatment; (c) Forty eight hours after QD treatment; and (d) effect of time on IL-1 β release for QD 565 and QD 655 when concentration and coating are held constant. Lower cases a-c: significant increase ($P < 0.05$) in IL-1 β release compared to (a) vehicle-treated controls, (b) the lower doses, or (c) other coatings within each QD size. Number 1 represents a significant effect of QD size on IL-1 β release when concentration, time, and coating are held constant ($P < 0.05$). (d) An asterisk denotes a significant effect of time on IL-1 β release when concentration and coating are held constant.

concentration on IL-6 release for QD 655 at 24 hours (Figure 5b). However, significant differences were apparent at 48 hours (Figure 5c). Carboxylic acid-coated QD 655 at the highest dose was elevated 2-fold compared to vehicle-treated controls, the lower dose, and PEG and PEG-amine-coated QD 655 at both doses. Comparison of the magnitude of IL-6 release for 20 nM carboxylic acid-coated QD 655 at 24 and 48 hours revealed a significant effect of time (Figure 5d).

The significant increase in IL-6 release at 24 hours for 20 nM carboxylic acid-coated QD 565 but not QD 655 indicated an effect of QD size on IL-6 release at 24 hours (Figure 5b). This effect was confirmed by statistical comparison. In contrast, at 48 hours, IL-6 release for carboxylic acid-coated QD 565 and QD 655 did not significantly differ (Figure 5c).

IL-8

The basal, control levels of IL-8 in the culture medium at 24 hours averaged $1,300 \pm 430$ and $3,000 \pm 600$ pg/ml at maximal response to QDs. Basal levels at 48 hours averaged $1,400 \pm 140$ and $2,800 \pm 480$ pg/ml at maximal response to QDs (data not shown).

Effects of dose and coating were observed for QD 565. At 24 hours, IL-8 release for 20 nM carboxylic acid-coated QD 565 was 2-fold greater than for vehicle-treated controls, the lower dose, and PEG and PEG-amine coatings at either concentration (Figure 6b). Forty-eight hours after treatment, the magnitude of IL-8 release for 20 nM carboxylic acid-coated QD 565 was unchanged (Figure 6d). Also, there was a slight but significant, dose-dependent increase in IL-8 release for 20 nM PEG-amine-coated QD 565 (Figure 6c).

Effects of coating, dose, and time on IL-8 release were observed in response to QD 655. There were no effects of coating or dose on IL-8 release for QD 655 at 24 hours (Figure 6b). By 48 hours, however, significant differences were apparent. IL-8 release for carboxylic acid-coated QD 655 at the highest dose was elevated over 2-fold compared to vehicle-treated controls, the lower dose, and PEG and PEG-amine-coatings at either dose. Also, there was a slight but significant dose-dependent increase in IL-8 release for 20 nM PEG-amine-coated QDs. Comparison of IL-8 levels for 20 nM carboxylic acid-coated QD 655 at 24 and 48 hours revealed a significant effect of time on IL-8 release (Figure 6d).

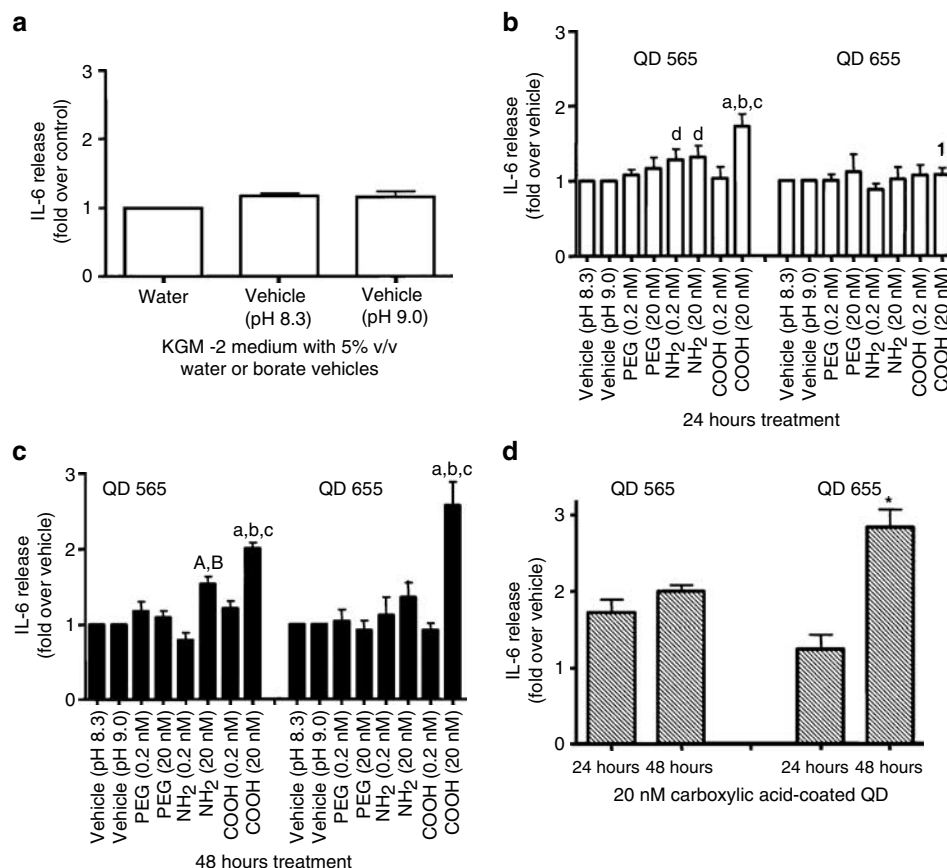


Figure 5. IL-6 release at 24 and 48 hours. (a) Effect of 5% (v/v) water or borate vehicle on IL-6 release at 24 hours. (b-d) HEKs were treated with 5% (v/v) of vehicle, QD 565, or QD 655 coated with PEG, PEG-amines, or carboxylic acids at 0.2 or 20 nM. (b) Twenty four hours after QD treatment; (c) Forty eight hours after QD treatment; and (d) effect of time on IL-6 release for QD 565 and QD 655 when concentration and coating are held constant. Lower cases a-c: significant increase ($P < 0.05$) in IL-6 release compared to (a) vehicle-treated controls, (b) the lower doses, or (c) other coatings within each QD size. (d) Not significantly different from 20 nM carboxylic acid-coated QDs. At 48 hours, upper case designate significant ($P < 0.05$) differences for 20 nM PEG-amine-coated QDs compared to (a) controls and the (b) lower dose. Number 1 represents a significant effect of QD size on IL-1 β release when concentration, time, and coating are held constant ($P < 0.05$). (d) An asterisk denotes an effect of time on IL-6 release when concentration and coating are held constant ($P < 0.05$).

An effect of size on IL-8 release for 20 nM carboxylic acid-coated QDs at 24 hours was indicated by the significant increase in the release of this cytokine for QD 565 but not QD 655 (Figure 6b). This effect was confirmed by statistical comparison. However, this effect of size did not extend to 48 hours, at which time the magnitude of IL-8 release for carboxylic acid-coated QD 565 and QD 655 did not differ (Figure 6c).

DISCUSSION

We used commercially available CdSe core/ZnS shell QDs of two sizes (QD 565 and QD 655) and three different surface coatings (PEG, PEG-amines, and carboxylic acids) to test the hypothesis that QDs would be differentially taken up by HEKs and cause cytotoxicity and inflammatory cytokine release depending on surface coating. We controlled for potential core-related effects by restricting these studies to QDs of the same core material with defined sizes and surface coatings. We found that all QDs tested localized to the interior of HEKs by 24 hours. However, cytotoxicity and proinflammatory cytokine release were dependent up surface

coating, a robust finding consistent across QDs of two different sizes (Table 1).

Previously, we reported that multi-walled carbon nanotubes were intracellularly localized in cytoplasmic vacuoles of HEKs (Monteiro-Riviere *et al.*, 2005b). The present study is the first to determine that a similar phenomenon extends to QDs in HEKs. Live cell imaging that eliminates fixation artifacts was used in conjunction with confocal laser scanning microscopy for this purpose. A low (2 nM), non-cytotoxic concentration of QDs was chosen to minimize uptake attributable to cell damage. All QDs localized intracellularly by 24 hours, which was surprising because these QDs were not "targeted" for cellular delivery. No evidence of colocalization was seen for QD 565 and QD 655 coated with PEG, which indicates that QDs with the same coating, but different core sizes, are differentially trafficked in HEKs. This result is consistent with a previous study, which showed that core size can influence the intracellular localization of QDs with the same coating (Lovric *et al.*, 2005a). In contrast, QD 565 and QD 655 coated with carboxylic acids or PEG-amines exhibited colocalization,

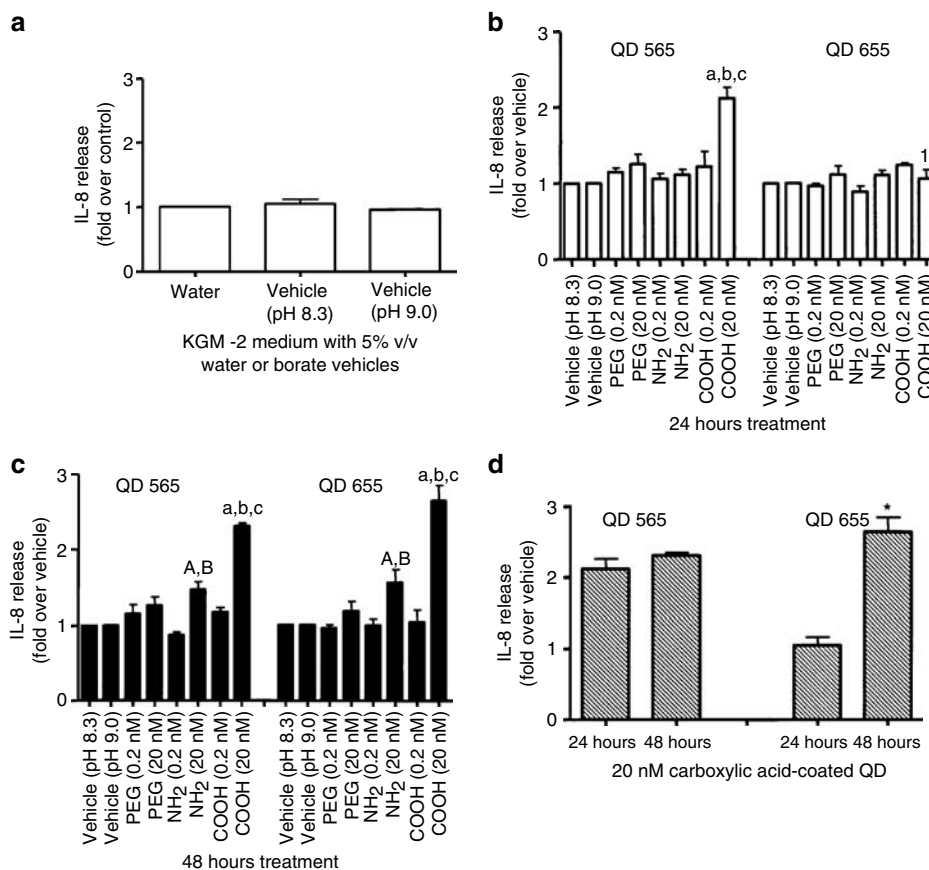


Figure 6. IL-8 release at 24 and 48 hours. (a) Effect of 5% (v/v) water or borate vehicle on IL-8 release at 24 hours. (b-d) HEKs were treated with 5% (v/v) of vehicle, QD 565, or QD 655 coated with PEG, PEG-amines, or carboxylic acids at 0.2 or 20 nM. (b) Twenty four hours after QD treatment; (c) Forty eight hours after QD treatment; and (d) effect of time on IL-8 release for QD 565 and QD 655 when concentration and coating are held constant. Lower cases a-c: significant increase ($P < 0.05$) in IL-8 release compared to (a) vehicle-treated controls, (b) the lower doses, or (c) other coatings within each QD size. At 48 hours, upper case designate significant ($P < 0.05$) differences for 20 nM PEG-amine-coated QDs compared to (A) controls and the (B) lower dose. Number 1 represents a significant effect of QD size on IL-8 release when concentration, time, and coating are held constant ($P < 0.05$). (d) An asterisk denotes an effect of time on IL-8 release when concentration and coating are held constant ($P < 0.05$).

which indicates cellular uptake and/or intracellular trafficking of QD 565 and QD 655 of the same coating by the same mechanisms. The result that QDs of different sizes and coatings localized to the interior of HEKs is of importance because it suggests a mechanism by which QDs may lodge in the avascular epidermis and escape removal by resident macrophages (Monteiro-Riviere and Inman, 2005).

We also observed nuclear localization of carboxylic acid and PEG-coated QDs by live cell confocal laser scanning microscopy, which was confirmed by TEM. Presently, it is not known if nuclear access of the QDs examined in this study would cause genetic damage in HEKs. There is one report, however, that CdSe core/ZnS shell QDs coated with biotin can nick supercoiled DNA in an *in vitro*, cell-free assay (Green and Howman, 2005).

Agglomeration and/or adsorption of proteins in the culture medium could modulate QD uptake into cells. TEM revealed agglomeration of QDs within HEKs. Agglomeration may occur in the culture medium as a result of nanoparticle interaction with media salts or proteins (Borm *et al.*, 2006),

which in the case of partially defined keratinocyte growth medium-2 (KGM-2) medium are human epidermal growth factor, insulin, transferrin and peptide hormones, and proteins from bovine pituitary extract. Alternatively, agglomeration may take place in cytoplasmic vacuoles. However, where agglomeration occurs, QDs were clearly available to the cell for uptake, as evidenced by the presence of these agglomerates in vacuoles and free in the cytoplasm for all QDs. Observation of free QDs in the cytoplasm is an important result, suggesting that QDs can escape cytoplasmic vacuoles or elude sequestration, which might allow interactions with cellular organelles or access to the nucleus. Occasionally, we noted the presence of lipid droplets peppered with electron-dense, PEG- and PEG-amine-coated QDs within the interior and along the periphery of the lipid droplet. Such lipids droplets are normally present in HEKs.

Cell viability dose-response assays showed significant cytotoxicity for PEG-amine- and carboxylic acid-coated QDs, but not PEG-coated QDs. These results were consistent

Table 1. Summary of QD effects in HEK.

	Cytotoxicity	IL-1 β	IL-6	IL-8
QD 565 _{PEG}				
24 h	NS	NS	NS	NS
48 h	NS	NS	NS	NS
QD 565 _{PEG-NH₂}				
24 h	NS	NS	NS	NS
48 h	+++	NS	+	+
QD 565 _{COOH}				
24 h	+++	+++	++	+++
48 h	+++	+++	++	+++
QD 655 _{PEG}				
24 h	NS	NS	NS	NS
48 h	+	NS	NS	NS
QD 655 _{PEG-NH₂}				
24 h	NS	NS	NS	NS
48 h	+++	NS	NS	+
QD 655 _{COOH}				
24 h	+++	+++	NS	NS
48 h	++++	++++	+++	+++

HEK, human epidermal keratinocytes; NS, not significant; QD, quantum dots.

Relative degree of effect within each column indicated by (+) or NS. Effects range from (+): statistically significant, but small in magnitude to (++++): statistically significant and large in magnitude.

for QDs of both sizes. PEG-coated QD 565 and QD 655 exhibited no appreciable cytotoxicity over 48 hours, but PEG-amine and carboxylic acid-coated QD 565 and QD 655 exhibited significant cytotoxicity within 48 hours at the highest dose. A modulatory role for exposure time in QD cytotoxicity was demonstrated by comparison of PEG-amine-coated QD 565 and QD 655 at 24 and 48 hours. No decreases in cell viability were observed at 24 hours, but cell viability had decreased significantly by 48 hours in both cases. We also observed a modest but significant effect of QD size on cytotoxicity when carboxylic acid-coated QD 655 exhibited an increase in cytotoxicity between 24 and 48 hours that was greater in magnitude than that observed for QD 565 with the same coating. The cytotoxicity of compositionally diverse QDs has been investigated in multiple cell lines and at multiple end points (Hardman, 2006; Monteiro-Riviere and Ryman-Rasmussen, 2006). Assessment of QD cytotoxicity at the MTT end point has been reported in several cell lines (Derfus *et al.*, 2004; Shiosahara *et al.*, 2004; Lovric *et al.*, 2005a,b), but the mechanisms underlying cytotoxicity have not been defined. Hypothesized mechanisms include oxidative stress as a result of QD-mediated reactive oxygen species production (Lovric *et al.*, 2005b) and

leaching of core metals into the culture medium (Derfus *et al.*, 2004). Metal leaching from the core is an unlikely mechanism of toxicity in the present study because core composition was controlled (i.e. these QDs differed only in surface coating) and the CdSe core of all QDs were “capped” with ZnS, which has been shown to attenuate leaching (Derfus *et al.*, 2004). Oxidative stress as a result of reactive oxygen species production cannot be ruled out, but does not directly address the clear effect of QD coating on HEK viability that we observed.

Before this work, inflammatory responses to QDs had not been explored. QDs and other nano-sized structures are expected to have high inflammatory potential owing to a large surface area to volume ratio (Oberdorster *et al.*, 2005). This expectation is based upon immune responses of the lung following exposure to airborne particulate matter, which has shown the greatest infiltration of immune cells for ultrafine (nano-sized) *versus* larger particles (Oberdorster, 2001). Immune responses of skin are substantially mediated by epidermal keratinocytes, which upon activation by environmental stimuli, secrete proinflammatory cytokines (Barker *et al.*, 1991; Nickoloff, 1991). Previous studies in our laboratory have shown that IL-8 is a suitable biomarker for cutaneous inflammation in response to diverse chemicals and chemical mixtures (Allen *et al.*, 2000, 2001; Chou *et al.*, 2002; Chou *et al.*, 2003; Monteiro-Riviere *et al.*, 2003;). The cytokines selected for the present study are broad markers for keratinocyte-mediated inflammation. Levels of all cytokines were normalized to the MTT viability data to account for reduced cell number resulting from cytotoxicity. We observed a robust effect of coating on cytokine release in response to QDs. This effect was consistent across two sizes of QDs and for three different cytokines. Carboxylic acid-coated QDs of both sizes increased release of IL-1 β , IL-6, and IL-8 2- to 5-fold over 48 hours, whereas PEG-coated QDs exhibited no increase in cytokine release and PEG-amine-coated QDs exhibited only a slight increase in IL-6 and IL-8. Modulatory effects of time and size on cytokine release for carboxylic acid-coated QDs were noted when carboxylic acid-coated QD 565 showed a 2-fold increase in IL-6 and IL-8 release at 24 hours that did not increase between 24 and 48 hours, but carboxylic acid-coated QD 655 did not cause significant elevations in IL-6 and IL-8 until 48 hours, at which time the magnitude of release was similar to that of QD 565. Also, both carboxylic acid-coated QD 565 and QD 655 showed a 2- to 3-fold increase in the release of IL-1 β at 24 hours. By 48 hours, however, the magnitude of IL-1 β release for QD 655 was 2.5-fold greater than that of QD 565, which remained at levels similar to the 24 hours time point. Thus, a 24 hours delay in maximal cytokine release for carboxylic acid-coated QD 655 relative for QD 565 was observed for all three cytokines. Also of consideration is the contribution of delivery vehicles to the inflammatory response. Although we saw no effects of the QD vehicles used herein on proinflammatory cytokine release, we have observed modulation of multi-walled carbon nanotubes-mediated IL-8 release in HEKs when delivered in pluronic surfactant vehicles (Monteiro-Riviere *et al.*, 2005a).

The present study suggests that the surface charge of the QDs, whether cationic or anionic, is an important variable for QD cytotoxicity and inflammation, with the cationic carboxylic acid-coated QDs showing the highest potential. This could be secondary to altered cellular localization of charged QDs or increased sensitivity of target site to these particles. This phenomenon, now recognized by this research, deserves further study. It is also of relevance for risk assessment in the light of evidence that QDs can penetrate the outer stratum corneum barrier of intact skin. Previously, we used these same QDs to show that topically applied QD 565 and QD 655 coated with PEG, PEG-amine, and carboxylic acids localized within the epidermis or dermis by 8 hours for all but carboxylic acid-coated QD 655, which localized primarily in the epidermis by 24 hours. Fluorescence intensity maps indicated a passive mechanism of penetration (Ryman-Rasmussen *et al.*, 2006). Recently, QDs have been shown to penetrate porcine skin within minutes when low frequency sonophoresis is applied (Paliwal *et al.*, 2006). Although the diffusion cell model utilized in both studies provided evidence that exposure to QDs can result in skin penetration, it does not address whether such exposures would cause skin irritation or damage. When our previous observation that carboxylic acid-coated QDs localize in the epidermis (Ryman-Rasmussen *et al.*, 2006) are considered in conjunction with the observation of cellular uptake in the present study, the tendency of carboxylic acid-coated QDs to localize in the epidermis may be secondary to the tendency for these QDs to preferentially accumulate in the keratinocyte. The present, *in vitro* study was conducted at QD concentrations 50- to 5,000-fold lower than the $1\ \mu\text{M}$ concentration we used for the dermal penetration study. The QDs tested herein are supplied at concentrations of up to $8.7\ \mu\text{M}$, which corresponds to human exposure concentrations that are up to 400- to 40,000-fold greater than those used in the present study. Thus, the concentrations at which we observed QD uptake, cytotoxic effects, and inflammatory cytokine release are arguably not supraphysiological, but within a reasonable range for human skin exposure to QDs as commercially supplied. Our results indicated potential for QDs coated with carboxylic acids in particular to cause cytotoxicity and cutaneous inflammation *in vivo*. Again, these are the same QDs in which we had observed primarily epidermal localization in our previous skin penetration study (Ryman-Rasmussen *et al.*, 2006). Cutaneous inflammation can be restricted to the site of entry or, as in the case of topical exposure to JP-8, also result in systemic modulation of immune function (Ullrich, 1999).

Our observation of QD uptake by HEKs at low, non-cytotoxic concentrations for all sizes and coatings begs the question of what the long-term effects of prolonged retention of QDs in HEKs might be. Particularly of importance is that uptake of QDs by HEKs was not selective because QDs were not "targeted" for cellular uptake by attachment to targeting peptides or antibodies, yet intracellular and intranuclear QD localization was observed. This suggests that QDs engineered to function as drug delivery vectors or diagnostic agents at other somatic sites may be non-selectively taken up by skin

cells, possibly resulting in side effects or cytotoxicity and immunogenicity in skin.

In summary, this study showed a clear role for surface coating in cytotoxicity and immunotoxicity that was consistent across two sizes. This study expanded our previous observations that dermal exposure to QDs results in skin penetration by showing that select QDs may pose an acute dermal hazard of cytotoxicity and inflammation, depending upon surface coating. Cytotoxic and inflammatory effects of QD surface coatings may extend to other families of engineered nanoparticles, which argues for the utility of QDs as important models for toxic responses of skin to nanoparticles with industrial and medical applications.

MATERIALS AND METHODS

Quantum dots

QDs with a CdSe core and ZnS shell of two different sizes were obtained from Quantum Dot Corporation/Invitrogen (Hayward, CA). QDs with fluorescence emission maxima at 565 nm (QD 565) have a spherical core/shell with a diameter of 4.6 nm. QDs with emission maxima at 655 nm (QD 655) have an ellipsoid core/shell with diameters of 6 nm (minor axis) by 12 nm (major axis) (Quantum Dot Corporation, 2005, personal communication). Each size was obtained with three different surface coatings: PEG, PEG-amine, or polyacrylic acid (carboxylic acids). These coatings are, respectively, neutral, positive, or negatively charged as supplied and at physiological pH. The hydrodynamic diameters of QD 565 as supplied were 35 nm (PEG), 15 nm (PEG-amine), and 14 nm (carboxylic acid). The hydrodynamic diameters (major axis) of QD 655 were 45 nm (PEG), 20 nm (PEG-amine), and 18 nm (carboxylic acid) (Quantum Dot Corporation, 2005, personal communication). QDs were sold as Qtracker[®] Non-targeted Quantum Dots (PEG) or Qdot[®] ITK[™] Amino (PEG) or Carboxyl QDs, respectively. QDs were supplied at concentrations ranging from 2 to $8.7\ \mu\text{M}$ in a 50 mM borate buffer of pH 9.0 (carboxylic acid-coated QDs) or pH 8.3 (PEG and PEG-amine-coated QDs).

Cell culture and QD treatment

Clonetics[™] cryopreserved primary HEKs were grown at 37°C and 5% CO₂ in KGM-2 medium according to the manufacturer's instructions (Cambrex Bio Science Walkersville Inc., Walkersville, MD) and were passaged twice before the start of experiments. Ninety-six-well microplates were coated with 100 μl of 0.1 mg/ml rat tail collagen Type I (BD Biosciences, Bedford, MA) in 20 mM acetic acid for 1 hour and rinsed with two volumes of calcium-free Hank's balanced salt solution (HBSS) before use. Diameter glass bottom culture dishes (35 mm) (WillCo Wells B.V., Amsterdam, The Netherlands) were coated with 1 ml of 0.1 mg/ml collagen for 1 hour and rinsed with two volumes of HBSS before use. Twenty-five cm² flasks were coated with 2 ml of 0.1 mg/ml collagen for 1 hour and rinsed with two volumes of HBSS before use. HEKs were plated at $1.5\text{--}2.0 \times 10^4$ cells/cm² and were grown to 40–60% confluency before QD treatment. As QDs of different coatings are supplied at different concentrations in borate buffers, it is critical that the final concentration of borate ions be the same after dilution into culture medium. QDs were vortexed for 1 minute to ensure complete suspension before dilution into borate buffer vehicle of the

appropriate pH (8.3 or 9.0) to 20 times the desired, final concentration. Diluted QDs were then immediately added to KGM-2 medium, and vortexed. The final concentration of borate in the medium was 0.5 mM and comprised 5% of the total volume. Vehicle-treated controls similarly contained 0.5 mM borate buffer of the appropriate pH comprising 5% of the total volume. Addition of water or borate vehicle (pH 8.3 or 9.0) to 5% (v/v) in KGM-2 does not change the pH of the medium.

Medium in 96-well plates (for MTT viability and cytokine assays) was replaced with 150 μ l of borate vehicle- or QD-containing medium. The final QD concentration was 0.2, 2, or 20 nM. QDs in culture medium were incubated with cells for 24 or 48 hours.

Dual label samples containing both QD 565 and QD 655 for live cell confocal studies were prepared to minimize the possibility of co-agglomeration. QDs were individually diluted in borate buffer vehicle of the appropriate pH and added to KGM-2 to a final concentration of 4 nM. Equal volumes of 4 nM QD 565 and QD 655 with the same surface coating (PEG, carboxylic acids, or PEG-amines) were mixed to a final concentration of 2 nM each of QD565 and QD 655. The medium in 35 mm glass bottom culture dishes was replaced with 2 ml of KGM-2 containing the borate buffer vehicle controls or 2 nM QD 565 and QD 655 and incubated for 24 hours before live cell confocal imaging.

HEKs were plated in 25 cm² flasks for TEM. Cells were treated with 2 nM QD 565 or QD 655 of each coating or with borate buffer vehicle controls for 24 hours before processing for TEM.

Laser scanning confocal microscopy of QDs in live HEKs

After 24 hours of dosing with QDs, 35 mm culture dishes were rinsed three times with 2 ml of HBSS and the medium replaced with 2 ml pre-warmed KGM-2. Culture dishes were mounted on a stage attached to an INC-2000 incubator system (20/20 Technology Inc., Wilmington, NC) with a 37°C humidified chamber and CO₂ line. QDs were imaged in live cells using a Nikon C1 confocal laser scanner connected to a Nikon Eclipse 2000E inverted fluorescence microscope with a \times 40 Plan Fluor ELWD (dry) objective (0.60 NA). QDs were excited with a 488 nm Ar laser line with filter-based emission channels of 565–615 nm (QD 565) and 630 nm and above (QD 655). Confocal-DIC images of HEK cells were obtained by excitation with a 633 nm HeNe laser line. Images were captured using Nikon EZ-C1 software (version 2.01.152). The full cellular thickness (approximately 30 μ m) was scanned in 1 μ m steps. Interior images were chosen to show QD subcellular localization. All imaging data are representative of three similar experiments conducted on different days.

TEM of QDs in HEKs

After 24 hours incubation with 2 nM QDs, medium was removed, cells were rinsed three times with two volumes of HBSS, and fixed in Trump's fixative at 4°C. HEKs were harvested by scraping into 1.5 ml microfuge tubes followed by centrifugation at 14,000 \times g. Cell pellets were embedded in 3% agar and post-fixed for 1 hour at 4°C in 1% osmium tetroxide. Samples were dehydrated in increasing concentrations of ethanol, cleared in acetone, and embedded in Spurr's resin. Thin sections were mounted on copper grids and examined on a Phillips Em208S TEM. Imaging data represent two separate experiments of 1–2 replicates for each QD size and coating.

MTT cell viability assays

An MTT assay was used to assess cell viability after 24 or 48 hours of QD treatment. Cell culture medium containing QDs was removed and 200 μ l of 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma-Aldrich, St Louis, MO) in KGM-2 was added to QDs and vehicle-treated wells. KGM-2 alone was added to duplicate control wells that contained vehicle-treated cells. HEKs were returned to the incubator for 3 hours. At the end of the incubation period, the medium was removed and HEKs were rinsed with 200 μ l of HBSS for 2 minutes. The HBSS was removed by aspiration, and 100 μ l of 70% isopropanol was added to each well and incubated for 60 minutes on a rotating platform to dissolve the MTT. Microplates were read at 550 nm in a Multiskan microplate spectrophotometer (Thermo LabSystems, Milford, MA) equipped with Ascent Software (version 2.6). The average background absorbance from control wells (KGM-2 without added MTT) was subtracted from MTT-treated samples and data were plotted as a percentage of the appropriate vehicle-treated control using Prism v. 4 software (Graph Pad Inc., San Diego, CA). Five replicates per data point were used for all treatments. Data are expressed as the means and SEs of two experiments (5% (v/v) water *versus* borate vehicle controls) or 4–6 experiments (QDs *versus* the appropriate borate vehicle-treated controls).

Cytokine release assays

Twenty-four hours after the addition of QDs to 96-well microplates ($N=5$ wells per treatment point), the culture medium was removed, pooled into a microfuge tube, and frozen at -80°C until assayed. On the day of the assay, samples were thawed and 50 μ l of each sample added (in duplicate) to a singleplex ELISA plate for human IL-8 and a multiplex ELISA plate for human IL-1 β , IL-6, IL-10, and tumor necrosis factor- α (BioPlex Cytokine Assays, BioRad Laboratories, Hercules, CA) and the ELISA performed according to the manufacturer's instructions. Samples were read on a BioPlex System (BioRad/Luminex) equipped with BioPlex software (version 4.0). Unknowns were quantified by linear regression to logarithmic standard curves. Cytokine levels were normalized relative to MTT absorbance. Data were expressed as fold over that of controls receiving the appropriate boric acid vehicle using Prism v. 4 software (Graph Pad Inc., San Diego, CA). Data are expressed as the means and standard errors of two experiments (5% (v/v) water *versus* borate vehicle-treated controls) or 4–5 experiments (QDs *versus* the appropriate borate vehicle controls).

Statistical analysis

A Kruskal-Wallis nonparametric analysis of variance was used to compare vehicle-treated cells to cells treated with water for cell viability and cytokine release assays. A one-way analysis of variance was used to compare the effect of concentration on cell viability and inflammatory cytokine release for QDs with size, surface coating, and time held constant. Significant differences were identified via a *post hoc* Tukey's test. An effect of time on cell viability and inflammatory cytokine release for each size/shape with surface coating and concentration held constant was identified by using a one-way analysis of variance followed by a *post hoc* Tukey's test. An effect of size/shape was similarly determined for the cell viability and cytokine release data by holding concentration and time constant. Significance for all values was set at $P<0.05$. All statistical analysis was performed using Prism v. 4 software.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Al Inman for assistance with TEM. This study was supported by USEPA-STAR Program No. RD83171501.

REFERENCES

- Allen DG, Riviere JE, Monteiro-Riviere NA (2000) Identification of early biomarkers of inflammation produced by keratinocytes exposed to jet fuels jet A, JP-8, and JP-8(100). *J Biochem Mol Toxicol* 14:231-7
- Allen DG, Riviere JE, Monteiro-Riviere NA (2001) Analysis of interleukin-8 release from normal human epidermal keratinocytes exposed to aliphatic hydrocarbons: delivery of hydrocarbons to cell cultures via complexation with alpha-cyclodextrin. *Toxicol In vitro* 15:663-9
- Barker JN, Mitra RS, Griffiths CE, Dixit VM, Nickoloff BJ (1991) Keratinocytes as initiators of inflammation. *Lancet* 337:211-4
- Borm P, Klaessig FC, Landry TD, Moudgil B, Pauluhn J, Thomas K *et al.* (2006) Research strategies for safety evaluation of nanomaterials, part V: role of dissolution in biological fate and effects of nanoscale particles. *Toxicol Sci* 90:23-32
- Chou CC, Riviere JE, Monteiro-Riviere NA (2002) Differential relationship between the carbon chain length of jet fuel aliphatic hydrocarbons and their ability to induce cytotoxicity vs. interleukin-8 release in human epidermal keratinocytes. *Toxicol Sci* 69:226-33
- Chou CC, Riviere JE, Monteiro-Riviere NA (2003) The cytotoxicity of jet fuel aromatic hydrocarbons and dose-related interleukin-8 release from human epidermal keratinocytes. *Arch Toxicol* 77:384-91
- Derfus AM, Chan WCW, Bhatia S (2004) Probing the cytotoxicity of semiconductor nanocrystals. *Nano Lett* 4:11-8
- Green M, Howman E (2005) Semiconductor quantum dots and free radical induced DNA nicking. *Chem Commun (Cambridge)* 1:121-3
- Hardman R (2006) A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. *Environ Health Perspect* 114:165-72
- Jaiswal JK, Mattoussi H, Mauro JM, Simon SM (2003) Long-term multiple color imaging of live cells using quantum dot bioconjugates. *Nat Biotechnol* 21:47-51
- Lovric J, Bazzi HS, Cuie Y, Fortin GR, Winnik FM, Maysinger D (2005a) Differences in subcellular distribution and toxicity of green and red emitting CdTe quantum dots. *J Mol Med* 83:377-85
- Lovric J, Cho SJ, Winnik FM, Maysinger D (2005b) Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death. *Chem Biol* 12:1227-34
- Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ *et al.* (2005) Quantum dots for live cells, *in vivo* imaging, and diagnostics. *Science* 307:538-44
- Monteiro-Riviere NA, Baynes RE, Riviere JE (2003) Pyridostigmine bromide modulates topical irritant-induced cytokine release from human epidermal keratinocytes and isolated perfused porcine skin. *Toxicology* 183:15-28
- Monteiro-Riviere NA, Inman AO (2005) Challenges for assessing carbon nanomaterial toxicity to the skin. *Carbon* 44:1070-8
- Monteiro-Riviere NA, Inman AO, Wang YY, Nemanich RJ (2005a) Surfactant effects on carbon nanotube interactions with human keratinocytes. *Nanomed: Nanotechnol Biol Med* 1:293-9
- Monteiro-Riviere NA, Nemanich RJ, Inman AO, Wang YY, Riviere JE (2005b) Multi-walled carbon nanotube interactions with human epidermal keratinocytes. *Toxicol Lett* 155:377-84
- Monteiro-Riviere NA, Ryman-Rasmussen JP (2006) Toxicology of nanomaterials. In: *Biological concepts and techniques in toxicology: an integrated approach*. (Riviere JE, ed), London: Taylor & Francis, 217-33
- Nickoloff BJ (1991) The cytokine network in psoriasis. *Arch Dermatol* 127:871-84
- Nirmal M, Dabbousi BO, Bawendi MG, Macklin JJ, Trautman JK, Harris TD, *et al.* (1996) Fluorescence intermittency in single cadmium selenide nanocrystals. *Nature* 383:802-4
- Oberdorster G (2001) Pulmonary effects of inhaled ultrafine particles. *Int Arch Occup Environ Health* 74:1-8
- Oberdorster G, Oberdorster E, Oberdorster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect* 113:823-39
- Paliwal S, Menon GK, Mitragotri S (2006) Low-frequency sonophoresis: ultrastructural basis for stratum corneum permeability assessed using quantum dots. *J Invest Dermatol* 126:1095-101
- Ryman-Rasmussen JP, Riviere JE, Monteiro-Riviere NA (2006) Penetration of intact skin by quantum dots with diverse physicochemical properties. *Toxicol Sci* 91:159-65
- Shiosahara A, Hoshino A, Hanaki K, Suzuki K, Yamamoto K (2004) On the cytotoxicity caused by quantum dots. *Microbiol Immunol* 48:669-75
- Ullrich SE (1999) Dermal application of JP-8 jet fuel induces immune suppression. *Toxicol Sci* 52:61-7